Cytokine-Induced CEACAM1 Expression on Keratinocytes Is Characteristic for Psoriatic Skin and Contributes to a Prolonged Lifespan of Neutrophils

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Carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1) is a cell-surface glycoprotein, belonging to the carcinoembryonic antigen family, expressed by human neutrophils, epithelial cells, activated T and NK cells. CEACAM1 is expressed as a cell-surface molecule with different isoforms or can be secreted as a soluble protein. Here, we show that keratinocytes in the outer epidermal layer of psoriatic skin express CEACAM1, unlike those in healthy skin or in cutaneous lesions of patients with atopic or nummular dermatitis. Stimulation of primary human keratinocytes or *in vitro* reconstituted epidermis with culture supernatants of activated psoriatic lesion-infiltrating T cells, IFN-γ or oncostatin M, but not IL-17, induced the expression of transcripts for the CEACAM1-long and -short isoforms and cell-surface CEACAM1, whereas soluble CEACAM1 was not produced. The uppermost layers of the epidermis in psoriatic lesions also contain neutrophils, a cell type with inflammatory and antimicrobial properties. Coculture of CEACAM1-expressing keratinocytes or CHO transfectants with neutrophils delayed spontaneous apoptosis of the latter cells. These results show that cytokine-induced cell-surface expression of CEACAM1 by keratinocytes in the context of a psoriatic environment might contribute to the persistence of neutrophils and thus to ongoing inflammation and the decreased propensity for skin infection, typical for patients with psoriasis.

Journal of Investigative Dermatology (2009) **129,** 671–681; doi:10.1038/jid.2008.303; published online 9 October 2008

INTRODUCTION

Carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1; Beauchemin *et al.*, 1999), formerly known as Biliary glycoprotein, C-CAM or CD66a, is a member of the Ig superfamily with a large spectrum of biological activities (reviewed in Gray-Owen and Blumberg, 2006). CEACAM1 regulates cell proliferation (Singer *et al.*, 2000), tumor growth (Hsieh *et al.*, 1995; Kunath *et al.*, 1995), apoptosis (Huang *et al.*, 1999; Singer *et al.*, 2005), angiogenesis (Ergun *et al.*, 2000), T-cell cytotoxicity (Morales *et al.*, 1999), neutrophil (Skubitz *et al.*, 1996) and dendritic cell (Kammerer *et al.*, 2001) functions, lumen formation of epithelial cells (Huang *et al.*, 1999), extracellular matrix protein-specific morphology and migration of endothelial cells (Müller *et al.*, 2005). Furthermore, It has a function in the adherence of activated neutrophils to cytokine-activated endothelium (Kuijpers *et al.*, 1992). Consistent with its multiple effects on cellular function, human CEACAM1 is expressed on a wide variety of cells in a wide range of normal and malignant tissues, including granulocytes, activated T and NK cells, transformed B cells, epithelial cells, extravillous intermediate trophoblast cells (Bamberger *et al.*, 2000), endothelial cells, and several types of carcinomas (Prall *et al.*, 1996; Markel *et al.*, 2002; Fahlgren *et al.*, 2003). CEACAM1 also functions as a microbial receptor and binds opacity protein-expressing *Neisseria* spp. (Virji *et al.*, 1996; Chen *et al.*, 1997; Gray-Owen *et al.*, 1997), outer membrane protein-expressing *Haemophilus* spp. (Virji *et al.*, 2000), and *E. coli* (Berger *et al.*, 2004), indicating its involvement in the recognition of bacteria and the regulation of bacterial colonization. Although the extracellular domains of CEACAM1 are heavily glycosylated, making up more than half of its molecular weight, the non-glycosylated-β sheet in the amino-terminal region of the molecule is the target for the various bacterial adhesion molecules and furthermore mediates the capacity of CEACAM1 to form homotypic intercellular interactions with itself, thereby serving as its own ligand (Oikawa *et al.*, 1992).

CEACAM1 mRNA is subject to extensive differential splicing (Barnett *et al.*, 1989). In human, 13 splice variants have been detected, potentially resulting in the formation of 11 different proteins, although the function of each splice

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Abbreviations: CEACAM1, carcinoembryonic antigen-related cellular adhesion molecule 1; CHO, Chinese hamster ovary; fMLP, N-formyl-Met-Leu-Phe; NHEK, normal human epidermal keratinocytes; OSM, oncostatin M; Th, T helper

Received 17 September 2007; revised 1 June 2008; accepted 23 June 2008; published online 9 October 2008

variant has not yet been validated. The two major splice isoforms of CEACAM1 consist of either three or four extracellular Ig-like domains that furthermore differ in their cytoplasmic tails, thereby yielding short (CEACAM1-3S and CEACAM1-4S) and long (CEACAM1-3L and CEACAM1-4L) transmembrane isoforms, respectively (reviewed in Beauchemin *et al.*, 1999; Gray-Owen and Blumberg, 2006). The relative expression levels of the L and S isoforms are not static, vary significantly between cell types and are furthermore dependent on the state of activation of the cells (Singer *et al.*, 2000; Greicius *et al.*, 2003; Scheffrahn *et al.*, 2005). Although expressed on a wide variety of epithelial cells (Prall *et al.*, 1996), little information is available on the expression of the CEACAM1 isoforms on human epithelial cells in the skin.

Keratinocytes are specialized cutaneous epithelial cells that form four distinct layers in the epidermis, the outer layer forming a selective barrier to the entry of foreign matter and infectious agents into the body. In response to skin injury, they release signaling molecules that modulate the expression of cell-surface receptors on immune competent cells, thereby modifying their cytoskeleton morphology and altering their proliferative and migratory capacities. Alteration of such complex interplay between epithelial and immune-competent cells is tightly associated with the pathogenesis of *Psoriasis vulgaris* a common autoimmune disease. The self-perpetuating activation of skin-infiltrating T lymphocytes contributes to the epidermal hyperproliferation and abnormal differentiation, characteristic for this disease, through the secretion of various soluble factors and cytokines (Lowes *et al.*, 2007).

The aim of this study was to determine the regulation of the expression of CEACAM1 and its isoforms by primary epidermal keratinocytes *in vitro* and *ex vivo*, as well as its possible involvement in the clinical manifestations of cutaneous immune-mediated inflammatory disease.

RESULTS

Keratinocytes in the outer epidermal layer of psoriatic lesions specifically express CEACAM1

The *in situ* expression of CEACAM1 in human skin biopsies was determined by immunohistochemistry, using a mAb suitable for *in situ* staining of CEACAM1, as validated by its reactivity with primary melanoma cells that strongly express this molecule (Figure 1a). Epidermal keratinocytes in any of the healthy skin samples ($n=9$) did not express CEACAM1, although a few dermal cells were positive (Figure 1b). We next analyzed whether epidermal keratinocytes under conditions of cutaneous inflammation express CEACAM1. Epidermal thickening and hyperproliferation of keratinocytes, indicative of psoriasis, were accompanied by a strong induction of CEACAM1 expression by these cells in the outer keratinocyte layer of all psoriatic skin samples tested (Figure 1c; $n=24$). Moreover, neutrophils present in the Munro-Saboureau microabscess, a typical feature of psoriatic skin, were strongly reactive with the anti-CEACAM1 mAb (Figure 1c). In contrast, no expression of CEACAM1 by hyperproliferative keratinocytes of supratumoral epidermis (Figure 1a)

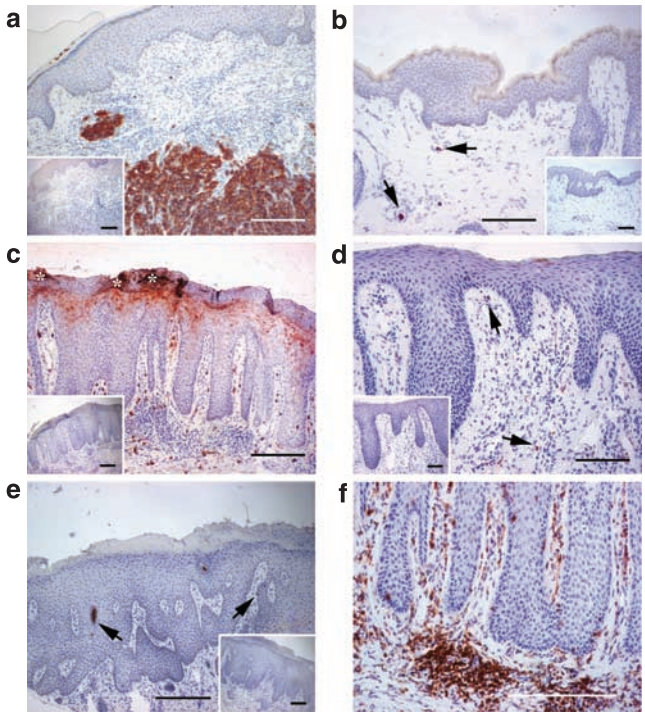

Figure 1. Keratinocytes in the outer epidermal layer of psoriatic lesions specifically express CEACAM1. CEACAM1 expression on cutaneous biopsies from individuals with (**a**) melanoma, from (**b**) healthy individuals, or patients with (**c**) psoriasis, (**d**) atopic dermatitis, or (**e**) nummular dermatitis was determined by immunohistochemistry analysis as described in 'Materials and Methods'. The presence of the Munro-Saboureau microabscess in the outer layer of psoriatic skin (**c**) is indicated by *. Arrows indicate CEACAM1 expression in the dermis of (**b**) healthy individuals or patients with (**d**) atopic or (**e**) nummular dermatitis. (**f**) CD3 expression in biopsies from patients with psoriasis. Controls are inserted in the relevant figures. Scale bars = 100 μm.

or by keratinocytes in the epidermis of atopic dermatitis lesions (Figure 1d; $n=17$) was observed, nor by those in the epidermis of five patients diagnosed with nummular dermatitis (Figure 1e). Although both psoriatic and atopic dermatitis lesions contained large numbers of skin-infiltrating T cells in the dermis, as indicated by positive staining with an anti-CD3 mAb (Figure 1f), few of these dermis-infiltrating cells were found to express CEACAM1 (Figure 1c). Moreover, no CEACAM1 expression by other cell types present in psoriatic lesional skin, such as $CD1a^+$ dendritic cells or Langerhans cells (data not shown), was observed. Taken together, these results demonstrate that keratinocytes in the outer epidermal layer of psoriatic lesions specifically express CEACAM1 and furthermore confirm that CEACAM1 is strongly expressed by epidermotropic neutrophils.

Cytokines produced by T cells that infiltrate psoriatic lesions induce CEACAM1 expression on normal primary keratinocytes and on reconstituted human skin *in vitro*

The absence of expression of CEACAM1 by keratinocytes in healthy skin, in contrast to its specific expression in the context of psoriatic inflammation, suggests that its expression might be subject to regulation by T cells that infiltrate this particular inflammatory environment. As the pathology of

psoriasis has traditionally been associated with the activity of T-helper (Th) type 1, IFN-γ-secreting T lymphocytes, primary skin-infiltrating T-cell lines generated from psoriatic lesions were analyzed for their capacity to induce the expression of CEACAM1 on normal human epidermal keratinocyte (NHEK). Culture supernatants from these T-cell lines, activated *in vitro* following stimulation with anti-CD3 and anti-CD28 mAbs, strongly induced the expression of CEACAM1 transcripts in NHEK with a maximal expression after 24 hours of incubation, as demonstrated by real-time RT-PCR (Figure 2a). Moreover, NHEK cultured for 48 hours in the presence of culture supernatants from activated psoriatic skin-infiltrating T cells were found to express CEACAM1 at their cell surface (Figure 2b). To determine whether T-cell-produced cytokines, and in particular IFN-γ, are involved in the regulation of CEACAM1 expression on keratinocytes, the culture supernatants were depleted of the latter cytokine before their addition to NHEK. The elimination of IFN-γ from these culture supernatants almost completely abrogated their CEACAM1 expression-inducing capacity, as shown by a strong decrease in CEACAM1 mRNA (Figure 2a), as well as the absence of protein expression at the cell surface (Figure 2b). Results from immunohistochemical analysis of NHEK, cultured for 48 hours in the presence of IFN-γ, directly confirmed the capacity of this cytokine to induce a strong expression of CEACAM1 on the latter cells (Figure 3a and b). These results were substantiated by western blotting analysis showing the expected molecular weight of about 140 kDa of the induced protein in extracts from IFN-γ-activated keratinocytes (Figure 3c).

We have recently demonstrated that oncostatin M (OSM) has potent modulatory effects on keratinocyte function (Boniface *et al.*, 2007). We therefore determined the capacity of this cytokine to induce the expression of CEACAM1 on either NHEK or on reconstituted human epidermis. Similar to the effects of IFN-γ, OSM-induced transcripts for CEACAM1 in NHEK and reconstituted epidermis (data not shown). In contrast, the addition of IL-17, a cytokine produced by a recently identified subpopulation of $CD4^+$ T cells called Th17 cells that are reportedly involved in the pathogenesis of

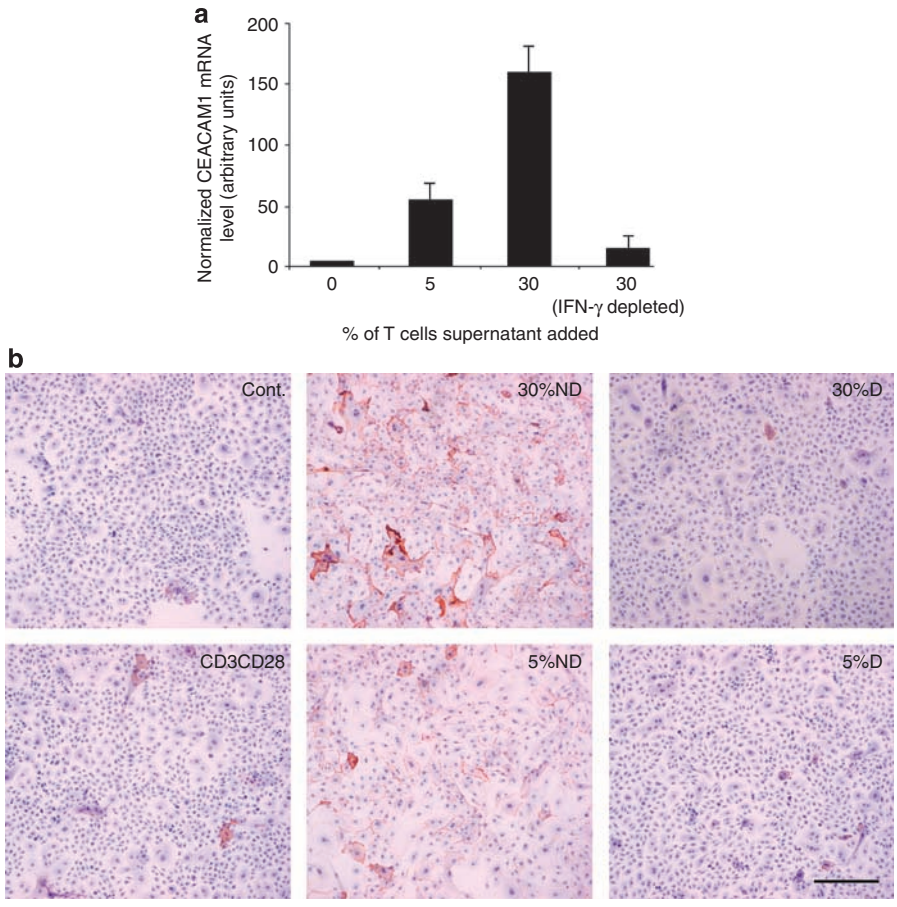

Figure 2. Culture supernatants of activated skin-infiltrating T cells induce expression of CEACAM1 transcripts and protein by NHEK in an IFN-γ-dependent manner. (**a**) NHEK were cultured with different dilutions of supernatants of *in vitro*-activated skin-infiltrating T cells, depleted or not for the presence of IFN-γ, and the expression of CEACAM1 transcripts was analyzed by real-time RT-PCR following 24 hours of incubation. (**b**) NHEK were cultured in medium only (Cont.) or in the presence of 30 or 5% culture supernatant, derived from psoriatic skin-infiltrating T cells, that had been activated for 24 hours *in vitro* with anti-CD3 and CD28 mAbs (30% ND and 5% ND). In addition, supernatants depleted for the presence of IFN-γ used at a concentration of 30% (30% D) or 5% (5% D) were added in parallel. As a control, NHEK were stimulated with anti-CD3 and CD28 mAbs (CD3CD28). Expression of CEACAM1 was determined by immunohistochemistry analysis after 48 hours of incubation. Scale bar = 100 μm.

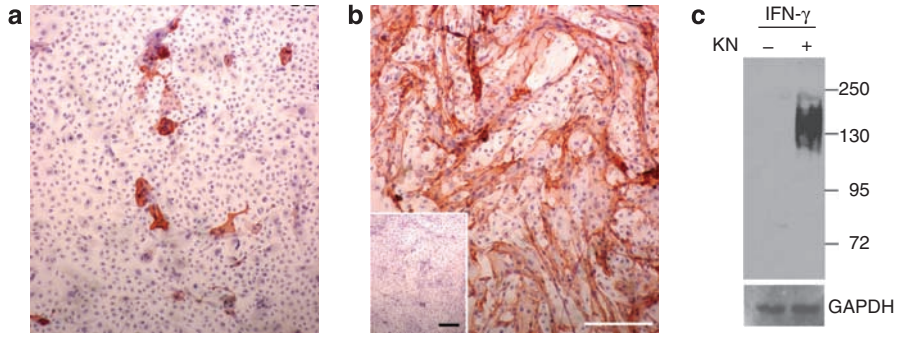

Figure 3. IFN-γ induces cell-surface expression of CEACAM1 by NHEK. NHEK were cultured for 48 hours in the (**a**) absence or (**b**) presence of IFN-γ. Cell-surface expression of CEACAM1 was determined by immunohistochemistry (**a**, **b**) or western blotting analysis (**c**). Negative control for immunohistochemical analysis is inserted in (**b**). Scale bar = 100 μm.

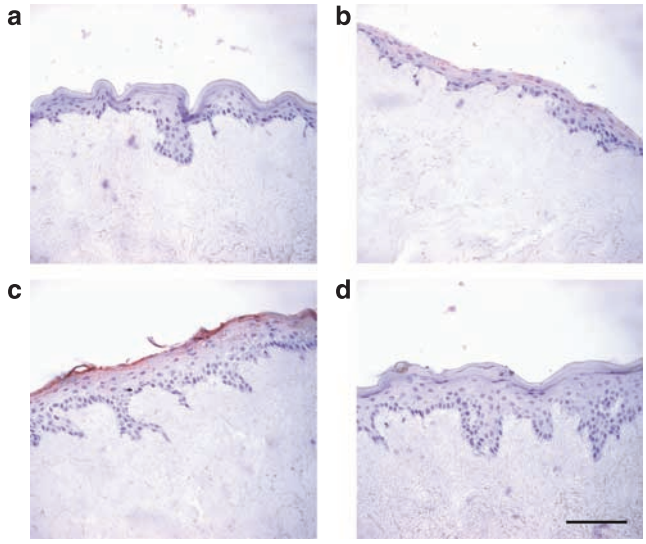

Figure 4. OSM induces cell-surface expression of CEACAM1 on human reconstituted epidermis. Human reconstituted epidermis was cultured for 48 hours in (**a**) the absence or (**b**) the presence of 30% T-cell-derived culture supernatant, with (**c**) OSM or (**d**) IL-17, both at a concentration of 10 ng ml^{-1}. The expression of CEACAM1 was determined by immunohistochemistry analysis. Scale bar = 100 μm.

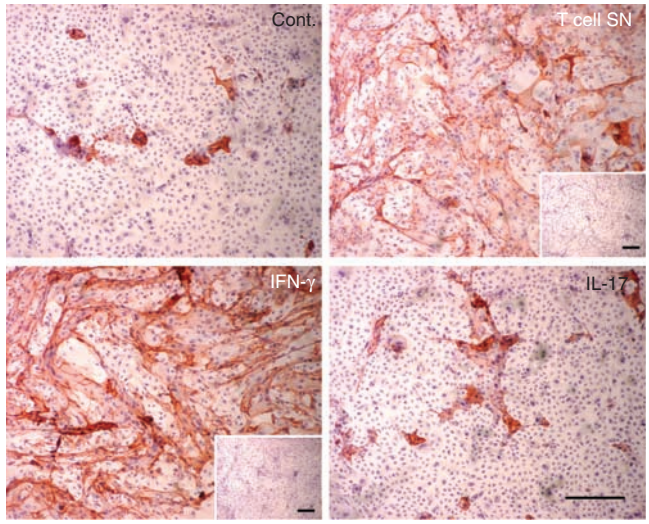

Figure 5. IL-17 does not induce CEACAM1 expression on NHEK. NHEK were cultured in medium only (Cont.) or in the presence of either a culture supernatant (30%, T-cell SN) of *in vitro*-activated psoriatic skin-infiltrating T cells, IFN-γ (10 ng ml^{-1}), or IL-17 and the expression of CEACAM1 was analyzed by immunohistochemistry after 48 hours of incubation. Negative controls for the two conditions that induce CEACAM1 expression are inserted in the figures. Scale bars = 100 μm.

several autoimmune and inflammatory disorders, did not result in the induction of CEACAM1 transcripts (data not shown). These results obtained at the transcriptional level were confirmed by immunohistochemical analysis showing that CEACAM1 expression was induced on reconstituted epidermis cultured in the presence of OSM, but not in the presence of IL-17 (Figure 4). The lack of CEACAM1 expression-inducing activity of IL-17 was also observed on NHEK (Figure 5). Culture supernatants of the activated T cells isolated from psoriatic lesions, used as a positive control in these experiments, induced CEACAM1 expression on both reconstituted skin (Figure 4) and NHEK (Figure 5).

Cytokine-activated keratinocytes express the CEACAM1-S and -L isoforms, but do not produce soluble CEACAM1

Various splicing events of CEACAM1 mRNA can give rise to the generation of three and four extracellular domains, in combination with long and short cytoplasmic tails, as well as soluble, isoforms. The ratio of CEACAM1-long and -short isoform expression differs according to cell type and activation state. We carried out both classic and real-time RT-PCR analysis in order to determine which CEACAM1 isoforms are induced on keratinocytes. Transcripts for the 3S, 3L, 4S, and 4L isoforms were detected in NHEK, mainly following culture with IFN-γ or OSM, thus confirming results showing cytokine-induced CEACAM1 protein expression on keratinocytes (Figures 2–4). It is of note that although IL-17 was inactive by itself, it synergized with OSM in the induction of each of the isoforms. Culture supernatants of psoriatic lesion-infiltrating T cells strongly induced all four CEACAM1 isoform transcripts, which was mainly dependent on the presence of IFN-γ, as shown by the depletion of the latter cytokine using a specific mAb. As is shown in Figure 6b, the primer combinations used in this study were specific for

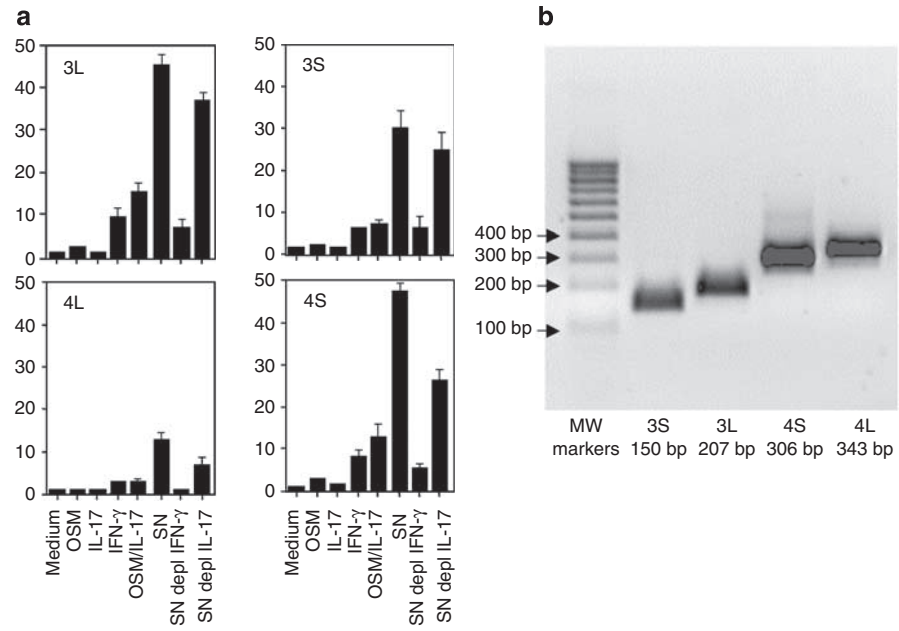

Figure 6. Cytokines produced by activated skin-infiltrating T cells induce the expression of CEACAM1-S and -L isoform transcripts on primary keratinocytes. (**a**) NHEK were cultured for 24 hours with a combinations of cytokines, each used at a concentration of 10 ng ml^{-1} or with a culture supernatant (30%) of *in vitro*-activated psoriatic skin-infiltrating T cells, in the presence or absence of neutralizing mAbs specific for IFN-γ or IL-17 and the expression of transcripts for the short (3S and 4S) and long (3L and 4L) isoforms of CEACAM1 was analyzed by real-time RT-PCR. (**b**) The specificity of the primers for the different isoforms was validated by classic PCR on NHEK, cultured in the presence of T-cell culture supernatant.

each of the four isoforms. In order to directly determine whether CEACAM1 isoform transcripts are overexpressed in psoriatic skin, quantitative PCR analysis was carried out on cutaneous samples taken from patients with psoriasis and compared with those from nonpsoriatic individuals ($n = 6$). However, no transcripts for either one of the four isoforms could be detected, neither in the psoriatic skin samples, nor in those from healthy nonpsoriatic skin. Finally, we determined the possibility that keratinocytes are able to produce the soluble isoform of CEACAM1, by analyzing secreted CEACAM1 levels in the culture supernatants of cytokine-activated NHEK using CEACAM1-specific ELISA. No soluble CEACAM1 was detectable in culture supernatants of NHEK, stimulated for 48 hours with either IFN-γ or with OSM (Table 1), under the same experimental conditions that induced the expression of CEACAM1 at the surface of these cells. As expected, *N*-formyl-Met-Leu-Phe (fMLP)- or GM-CSF-activated neutrophils, used as a positive control, produced soluble CEACAM1.

Table 1. Activated human keratinocytes do not produce soluble CEACAM1

Culture conditions[1]	CEACAM1 (ng ml^{-1})[2]
NHEK	
Medium	0.57 ± 0.42
IFN-γ	1.21 ± 1.15
OSM	0.95 ± 0.86
Neutrophils	
Medium	0.38 ± 0.09
fMLP	10.26 ± 0.31
GM-CSF	27.52 ± 0.13

CEACAM1, carcinoembryonic antigen-related cellular adhesion molecule 1;NHEK, normal human epidermal keratinocytes; OSM, oncostatin M; fMLP, *N*-formyl-Met-Leu-Phe.
[1]NHEK were cultured in medium alone or in the presence of IFN-γ or OSM (both at 10 ng ml^{-1}). As a control, freshly isolated peripheral blood neutrophils were cultured in medium only or stimulated with fMLP (10^{-8} M) or with GM-CSF (200 ng ml^{-1}).
[2]After 24 h, the culture supernatants were analyzed, by specific ELISA, for the presence of soluble CEACAM1. Results of four independent experiments (mean ± SD).

Activated keratinocytes delay neutrophil apoptosis by homotypic CEACAM1 interactions

Active *P. vulgaris* skin lesions are characterized by the presence of neutrophils in the typical Munro-Sabouraeu microabscess in the outer layer of the epidermis. As neutrophils rapidly undergo apoptosis after having left the circulation, we investigated whether CEACAM1-mediated homotypic interactions between keratinocytes and neutrophils might contribute to the persistence of the latter cells in this particular skin disease. Early and late apoptotic stages of neutrophils, cultured in the presence or absence of CEACAM1-expressing NHEK, were analyzed by measuring Annexin-V binding and PI incorporation, respectively, using flow cytometry. After 24 hours of coculture with NHEK about 55% of the neutrophils were in an early apoptotic state (Figure 7c), which was similar to that of neutrophils cultured in medium only (Figure 7a). In contrast, coculture of

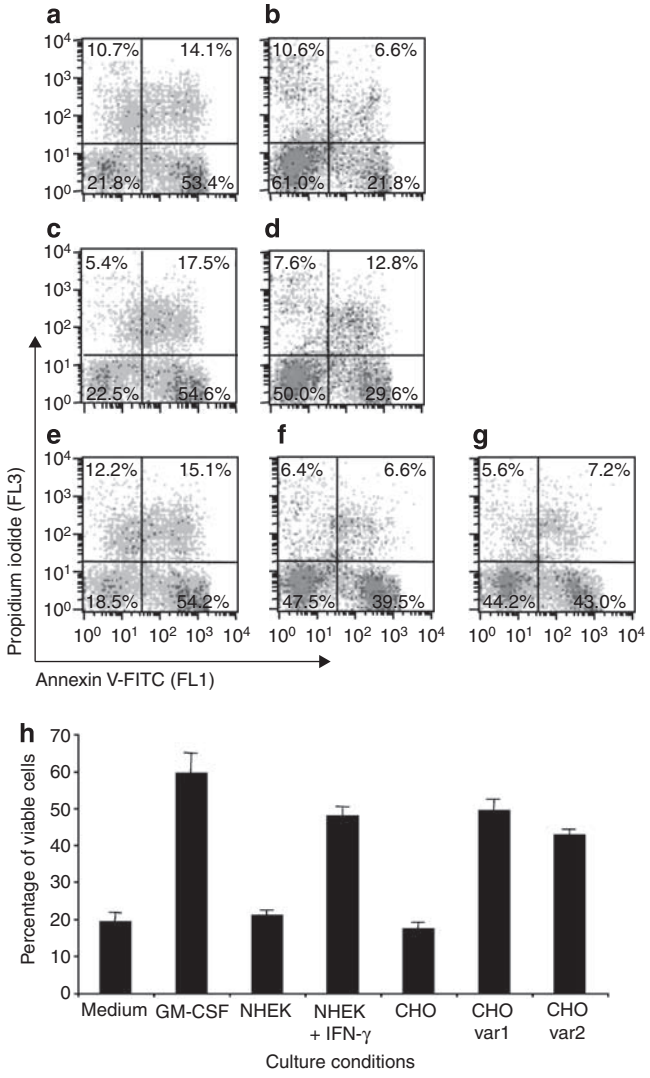

Figure 7. IFN-γ-induced expression of CEACAM1 on keratinocytes protects neutrophils from spontaneous apoptosis. (**a**) One million of freshly isolated peripheral blood neutrophils were cultured for 24 hours in medium alone or (**b**) 200 ng ml^{-1} GM-CSF or were cocultured with either NHEK that had been preincubated for 48 hours (**c**) in medium, (**d**) with 10 ng ml^{-1} IFN-γ, or with (**e**) wild type CHO cells or with CHO cells expressing (**f**) the long or (**g**) the short isoform of CEACAM1. Frequencies of viable and early or late apoptotic cells were determined by measuring Annexin-V binding (*x* axis) and PI incorporation (*y* axis) using flow cytometry. Results shown in (**a**) to (**g**) are representative of three independent experiments with neutrophils from different donors and the percentages of cells in the quadrants are indicated in each graph. (**h**) Percentages of viable cells are represented as the mean ± SD.

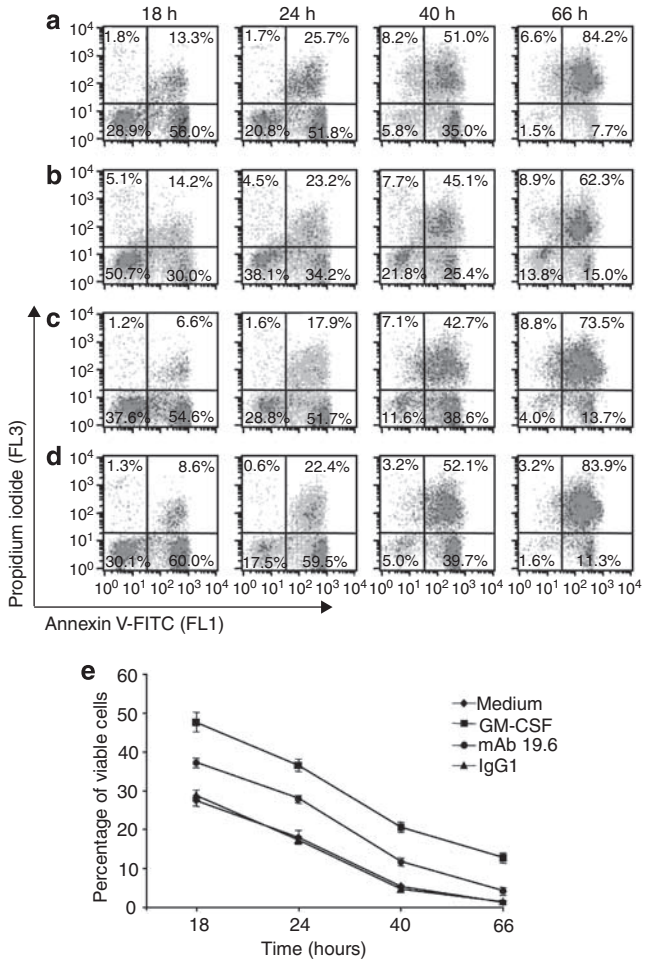

Figure 8. CEACAM1 engagement delays neutrophil apoptosis. One million of freshly isolated peripheral blood neutrophils were either cultured (**a**) in medium only, (**b**) with 200 ng ml^{-1} GM-CSF, (**c**) with 30 μg ml^{-1} of the anti-CEACAM1 mAb 19.6, or (**d**) with 30 μg ml^{-1} of an isotype control mAb for various periods of time. Frequencies of viable and early or late apoptotic cells were determined by measuring Annexin-V binding and PI incorporation using flow cytometry. Representative results from four independent experiments with neutrophils from different donors. Percentages of cells in the quadrants are indicated. (**e**) Kinetics of viable cell frequencies are expressed as the mean ± SD of the values obtained in the four experiments.

neutrophils with NHEK that had been preincubated with IFN-γ reduced the level of early apoptotic cells (30%) and increased numbers of viable cells thus suggesting a protective effect of CEACAM1-expressing NHEK (Figure 7d and h). To more directly demonstrate the involvement of CEACAM1-dependent interactions in this process, neutrophils were cocultured with Chinese hamster ovary (CHO) cells expressing either the 4S or the 4L isoforms of CEACAM1. Both transfectants exerted a protective effect on neutrophils, similar to that observed with CEACAM1-expressing NHEK (Figure 7f and g) as compared to the wild-type nontransfected CHO cell line (Figure 7e). GM-CSF, a factor known for its ability to delay neutrophil apoptosis by promoting the growth of these cells, which was added as a positive control, significantly delayed spontaneous cell death after 24 hours of culture (Figure 7b).

Finally, in order to determine whether the CEACAM1-mediated effects were long lasting, neutrophils were cultured for various periods of time in the presence of an anti-CEACAM1 mAb or with GM-CSF. As expected, the protective effect of GM-CSF was maintained until 66 hours of culture, as a high percentage of viable neutrophils was still observed at that time point (Figure 8b and e), as compared to the experimental conditions using medium alone (Figure 8a and e). CEACAM1 engagement by means of the addition of the 19.6 mAb also delayed spontaneous neutrophil apoptosis at

least until 40 hours after the initiation of the cultures (Figure 8c) and even after 66 hours of culture, 4% of neutrophils were still viable (Figure 8e), whereas the addition of an isotype control IgG was ineffective (Figure 8d and e). Taken together, these results indicate that CEACAM1-mediated homotypic interactions between cytokine-activated keratinocytes and neutrophils result in a delay in the spontaneous apoptosis in the latter cells, although they do not prevent late apoptotic events.

DISCUSSION

CEACAM1 is a well-studied member of a family of cell-surface antigens that is involved in intercellular adhesion and intracellular signaling events in normal as well as in cancer cells and that furthermore has been reported to be important as a regulatory receptor in immune responses of both myeloid and lymphoid cells. Results from extensive histochemical analysis on human tissue have shown that CEACAM1 is expressed on most, if not all, types of epithelial cells (Prall *et al.*, 1996). However, we report here that keratinocytes in normal, healthy skin do not express CEACAM1 and that its expression is specifically upregulated under conditions of inflammation in the outer epidermal layer of psoriatic skin. CEACAM1 expression on activated keratinocytes is not typical for cutaneous inflammation in general, as it could not be detected in the epidermis of patients with atopic dermatitis. Its specificity for psoriatic inflammation is furthermore underscored by results showing that cutaneous tissue from patients with nummular dermatitis does not express CEACAM1. Therefore, its discrete expression pattern makes CEACAM1 a useful biological and immunohistochemical marker for the diagnosis of psoriasis.

The differential expression of CEACAM1 on keratinocytes in cutaneous lesions of atopic dermatitis patients and those in psoriatic lesions may reflect the underlying pathogenesis of these skin disorders. It is generally accepted that the immune response in atopic dermatitis is mediated by Th2 lymphocytes, contributing to the high IgE levels and eosinophilia characteristic of this condition (Leung *et al.*, 2004), whereas, in contrast, Th1 cells are important in the pathogenesis of psoriasis (Bowcock and Krueger, 2005). Moreover, recently a separate lineage of Th cells has been identified, referred to as Th17 cells that, by the production of IL-17, as well as IL-22, is involved in the induction of autoimmune and inflammatory disorders, such as psoriasis, inflammatory bowel disease, and rheumatoid arthritis (review in Bettelli *et al.*, 2007). Therefore, the specific overexpression of CEACAM1 on keratinocytes in psoriatic lesions is most likely the consequence of the particular cytokine production profile of these skin-infiltrating T-lymphocyte subpopulations. Indeed, the induction of CEACAM1 expression on keratinocytes was found to be induced by culture supernatants of these cells and to involve the activity of the classical Th1 cytokine IFN-γ. Moreover, OSM, recently reported by us and others to induce the expression of several genes associated with the pathogenesis of psoriasis (Wolk *et al.*, 2006; Boniface *et al.*, 2007), was found to upregulate CEACAM1 expression on primary keratinocytes. In contrast however, although IL-17 strongly induces the expression of transcripts for β-defensins by keratinocytes (Boniface *et al.*, 2007), it does not have CEACAM1 expression-inducing capacity, as determined either by the direct addition of this cytokine to cultures of NHEK (Figure 5) or to reconstituted human epidermis (Figure 4).

The gene encoding CEACAM1 consists of 9 exons that can be alternatively spliced to generate a total of 11 isoforms, including 3 soluble forms referred to as CEACAM1-4C1, CEACAM1-3, and CEACAM1-3C2 (review in Gray-Owen and Blumberg, 2006). Although CEACAM1 molecules are able to form homotypic interactions, it is not clear whether the secreted CEACAM1 isoforms act as antagonists, thereby interfering with membrane-bound CEACAM1 interactions, or whether they are also capable to mediate CEACAM1-dependent signaling following interaction with membrane-bound CEACAM1. Results from a recent study have indicated that activated rat granulocytes secrete soluble CEACAM1 (Singer *et al.*, 2005). Whereas human neutrophils, activated with fMLP were found to secrete soluble CEACAM1 (Table 1), activated keratinocytes, in spite of expressing membrane-bound CEACAM1, did not secrete detectable levels of the soluble isoform of CEACAM1 which therefore does not seem to be involved in human keratinocyte-mediated biological effects.

In spite of the preferential expression of CEACAM1 in a psoriatic inflammatory environment, there is however little evidence for a direct implication in the pathogenesis of psoriasis, as CEACAM1 is not expressed by keratinocytes that constitute the main layer of cells undergoing hyperproliferation and abnormal differentiation in psoriatic lesions. We therefore hypothesize that CEACAM1 could be involved in another clinical aspect of psoriatic inflammation, that is in the presence of neutrophils in the Munro-Sabouraud microabscesses which are located in the same outer epidermal layer as the CEACAM1-expressing keratinocytes in psoriatic lesions. Neutrophils, a cell type with strong inflammatory and antimicrobial properties rapidly undergoes spontaneous apoptosis once these cells have left the circulation. The results of the present study show that coculture of cytokine-activated keratinocytes with neutrophils delays spontaneous apoptosis of the latter cells thereby confirming a, previously published, similar effect on rat granulocytes, following engagement of CEACAM1 by a specific mAb (Singer *et al.*, 2005), and thus suggest that homotypic CEACAM1-dependent interactions are likely to contribute to a prolonged presence of neutrophils in psoriatic skin.

As expected, GM-CSF was found to strongly delay neutrophil apoptosis. It is has been reported that neutrophils cultured in the presence of GM-CSF expressed increased levels of CEACAM1 transcripts, as well as CEACAM1 protein at their cell surface (Kobayashi *et al.*, 2005). As epidermal keratinocytes have been reported to produce GM-CSF following activation (Kupper *et al.*, 1988), they might contribute to optimal homotypic CEACAM1 interactions, not only between activated keratinocytes and neutrophils, but also between neutrophils themselves, thus resulting in enhanced and sustained inflammatory responses. It can however not totally be excluded that cell-surface molecules

other than CEACAM1 play a role in this process. The persistence of neutrophils in psoriatic lesions might, in part, explain the difference in the propensity for skin infection between patients with psoriasis and atopic dermatitis. Despite the presence of defective skin barriers in both types of skin diseases, about one-third of all patients with atopic dermatitis suffer from frequent skin infections (Kupper *et al.*, 1988), a complication only encountered in a minority of psoriasis patients. This difference in susceptibility to skin infection in patients with psoriasis has been associated with the development of an adequate cutaneous innate immune response, such as the secretion of β-defensins (Ong *et al.*, 2002), psoriasin or other antimicrobial peptides (Nomura *et al.*, 2003) by keratinocytes which is also under the control of cytokines produced by activated T cells in particular IL-17 and OSM (Boniface *et al.*, 2007). Neutrophils too, in particular through the generation of reactive oxygen species, are important in the killing of pathogenic microbial organisms and their persistence at site of possible infection, as a result of CEACAM1-mediated delay of apoptosis, may therefore be causally associated with lesser bacterial infection in patients with psoriasis. CEACAM1 has been described as a receptor for *Hemophilus influenza* and *Neisseria* spp. (Leusch *et al.*, 1991; Chen *et al.*, 1997; Muenzner *et al.*, 2000). The involvement of CEACAM1 in the colonization of these microbial organisms together with its specific expression by keratinocytes in psoriatic lesions therefore seems to be in contradiction with an increased resistance of psoriasis patients to microbial infection. At present however, the number of different microbial organism described to bind to CEACAM1 is rather limited and those reported in the literature have not been linked to the cutaneous infection observed in patients with atopic dermatitis. Therefore, a possible role for CEACAM1 in the colonization of microbial organisms in cutaneous disorders characterized by ruptured skin remains to be proven.

It is furthermore shown in this study that expression of transcripts for all CEACAM1-L and -S isoforms is induced in human keratinocytes by IFN-γ and OSM, although with different efficiency, and with the strongest effects displayed by IFN-γ. Nevertheless, at present, the functional significance of the expression of these isoforms by activated keratinocytes remains elusive. Finally, in spite of the association between expression of CEACAM1 transcripts and protein in cytokine-stimulated keratinocytes, no expression of any of the CEACAM1 isoforms transcripts was detected in the cutaneous biopsies of psoriatic patients. The most likely explanation is that their expression is below levels of detection, because of the restriction of CEACAM1 expression to the outer epidermis layer of psoriatic skin, resulting in the dilution of the mRNA's in the entire samples.

Taken together, our results show that keratinocytes in the outer layer of the epidermis in psoriatic lesions express CEACAM1, thus forming a biological marker for the diagnosis of this disease, and that the expression of the CEACAM1-L and -S isoforms is induced *in vitro* on human keratinocytes by IFN-γ and OSM, both produced by T cells that infiltrate psoriatic lesions. Although CEACAM1-mediated signaling does not seem to be associated with abnormal keratinocyte function in psoriasis, cytokine-induced cell-surface expression of CEACAM1 on keratinocytes in the context of a psoriatic environment might contribute to the persistence of neutrophils and thus ongoing inflammation, as well as to the decreased propensity for skin infection, typical for patients with psoriasis.

MATERIALS AND METHODS

Anti-CEACAM1 mAbs

The 19.6 mAb (IgG1) was generated following the immunization of 12 weeks old female BALB/c mice with the skin-derived T-cell clone BOY-JF.161 (Lecart *et al.*, 2001) and fusion of mouse spleen cells with the myeloma line X-63 Ag-8. Culture supernatants of growing hybridomas were tested by immunofluorescence and flow cytometry and the corresponding mAb was selected for reactivity with a panel of skin-derived T-cell clones, absence of reactivity with $CD4^+$ peripheral blood T cells and for suitability in western blotting and immunoprecipitation procedures. The molecule recognized by the 19.6 mAb was identified by mass spectrometry and sequencing of tryptic peptides. A Mascot search of 7 out of the 10 peptides generated in this way permitted to identify with a probability score of $P<0.001$ a peptide with the amino-acid sequence QIVGYAIGTQ-QATPGPANSGR that is characteristic of human CEACAM1 (P13688). Moreover, the 19.6 mAb recognizes the expected 115 kDa glycoprotein on a 64 kDa protein backbone on activated T cells, as shown by western blotting analysis (results not shown). Finally, formal proof of the specificity of the 19.6 mAb for the extracellular domain of CEACAM1 was provided by the observation that CHO cells, transfected with cDNA encoding either the 4L or the 4S isoform of CEACAM-1 (see below), stained equally well with both this mAb and the commercially available CEACAM1-specific mAb MAB2244 (R&D Systems Europe, Lille, France; results not shown). On the basis of sequence alignment of the transfected cDNA, it can be concluded that the 19.6 mAb recognizes an epitope within the sequence of the IgV-like domain between amino-acid residues 79 and 98 (results not shown).

Cells, cell culture, and cytokines

Primary T-cell lines were generated from cutaneous punch biopsies of lesional skin of patients with *P. vulgaris* that had been cultured in the presence of the anti-CD3- and anti-CD28 mAb-coated T-cell Expander beads (Invitrogen Life Technologies, Cergy-Pontoise, France), according to the manufacturer's recommendations, and expanded for 10–14 days in Yssel's medium (Yssel *et al.*, 1984), supplemented with 1% human serum (Etablissement Français du Sang, Lyon, France) and 20 ng ml^{-1} rIL-2 (Diaclone Research, Besançon, France) before use in experiments. The use of human tissue was conducted according to the Declaration of Helsinki principles, after written informed patient's consent and was approved by the ethical committee of the University Hospitals of Montpellier. NHEK were isolated from foreskin, as reported in the literature (Rheinwald and Green, 1977) and were cultured in keratinocyte serum-free medium (K-SFM; Invitrogen Life Technologies), supplemented with epidermal growth factor (5 ng ml^{-1}) and bovine pituitary extract (50 μg ml^{-1}; Invitrogen Life Technologies) at 37 °C, 5% CO_2 in a humidified incubator. Peripheral blood neutrophils were isolated from venous blood of healthy volunteers

by percoll gradient centrifugation, resuspended in Hank's balanced salt solution and immediately used in experiments. CHO cells transfected with cDNA encoding the long (NCBI database, NM001712) or short (NM001024912) isoforms of CEACAM1 were cultured in Iscove's modified Dulbecco's medium (IMDM), supplemented with 10% fetal calf serum (Biowest, Nuaille, France) and 300 μg ml^{-1} of G418 (Invitrogen Life Technologies). The following recombinant cytokines were used IFN-γ, IL-17, and OSM (final concentration 10 ng ml^{-1}; R&D Systems).

Reconstituted human skin

Reconstituted human skin was generated as described previously (Moles *et al.*, 1994). Briefly, keratinocyte suspensions (2×10^5 cells in 15 μl) were seeded on the center of a dead, de-epidermized dermis and grown at the air–liquid interface on a metallic support for 2 weeks at 37 °C, 5% CO_2 in a humidified incubator. Culture medium consisted of Ham's F12/DMEM (1/3; v/v), supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% fungizone (Invitrogen Life Technologies), 0.4 μg ml^{-1} hydrocortisone, 5 μg ml^{-1} insulin, 10^{-10} M cholera toxin, and 10 ng ml^{-1} EGF (Sigma-Aldrich, Saint-Quentin-Fallavier, France). The culture medium was supplemented with the various cytokines during the 2 weeks of reconstruction.

Generation of T-cell culture supernatants

Primary T-cell lines were washed extensively in serum-free medium without any supplements and were cultured at a concentration of 2×10^6 cells ml^{-1} in the same culture medium in the presence of plate-bound anti-CD3 (SPV-T3b; Beckman-Coulter, Roissy, France; coated for 4 hours at 37 °C at a concentration of 10 μg ml^{-1} in phosphate-buffered saline, PBS) and anti-CD28 (L293; BD Biosciences, Le Pont de Claix, France; 1 μg ml^{-1}) mAbs. Controls consisted of culture medium incubated only with the latter mAbs and were used to evaluate possible nonspecific activation of the keratinocytes. Culture supernatants were collected after 24 hours of culture and following removal of remaining cells by centrifugation, aliquoted and stored at −80 °C before use. Depletion of IFN-γ from the culture supernatants was carried out as follows: 250 μl of goat-anti-mouse IgG Ab-coated magnetic beads (Dynal, Compiègne, France) were incubated with 100 μl of the neutralizing anti-human IFN-γ mAb BB-1 (1 mg ml^{-1}; Diaclone Research) or anti-human IL-17 mAb (1 mg ml^{-1}; R&D Systems) for 30 minutes at 4 °C under orbital shaking. After washing with PBS containing 2% fetal calf serum, the mAb-coated beads were incubated with 1 ml of T-cell-derived culture supernatant for 30 minutes at 4 °C under orbital shaking. After removal of the beads by a magnetic device (Invitrogen Life Technologies), the culture supernatant was used directly in experiments. This procedure allows the complete and specific removal of a cytokine from the culture supernatant, as determined by cytokine-specific ELISA following depletion (unpublished observations).

Immunofluorescence and flow cytometry analysis

All immunofluorescence and flow cytometry procedures were carried out as described (Scheffold *et al.*, 2002). In addition to the anti-CEACAM-1 mAbs referred to above, the following (m)Abs and control IgG were used: nonconjugated anti-CD3 mAb SPV-T3b, nonconjugated anti-CD25 mAb, and nonconjugated mouse IgG1 as isotype-specific negative control (BD Biosciences); PE-conjugated anti-CD4 and a FITC-conjugated goat-anti-mouse IgG Ab (Caltag, Le Perray-en-Yvelines, France). Cells were analyzed on a FACSCalibur flow cytometer equipped with Cellquest software (BD Biosciences).

RNA isolation and quantitative RT-PCR analysis

Total cellular RNA from keratinocytes was extracted using Tri-Reagent (Sigma-Aldrich; T9424) following the manufacturer's protocol. Total RNA was reverse-transcribed using the primer oligo(dT) and Superscript II enzyme (Invitrogen Life Technologies). cDNAs, were subsequently analyzed by quantitative real-time PCR using the LightCycler system (Roche Diagnostics, Meylan, France), as described originally by Wittwer *et al.* (1997), as well as classic PCR. Primer sequences were: *Homo sapiens* CEACAM1-4S (NM001024912): sense—AAACCAGAGTCTCCCGTCCT, antisense—TGGAGTGGTCCTGAGCTGCCG; CEACAM1-4L (NM001712): sense—AAACCAGAGTCTCCCGTCCT, antisense—TTGTGCTCTGTGAGATCACGC or antisense—GTGGTTGGAGACTGAGGGTTTG; CEACAM1-3S (AY766113): sense—TCACTGATAATGCTCTACCACAAGA, antisense—TGGAGTGGTCCTGAGCTGCCG: CEACAM1-3L (X14831): sense—TCACTGATAATGCTCTACCACAAGA, antisense—TTGTGCTCTGTGAGATCACGC or antisense—GTGGTTGGAGACTGAGGGTTTG. The different combinations of these primers were used to amplify the individual CEACAM1 isoforms. The calculated amount was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Immunohistochemical staining

Punch biopsies from lesional skin of patients with psoriasis ($n = 24$) or with atopic dermatitis ($n = 17$) were collected, immediately embedded in OCT-compound (Miles, Zoeterwoude, the Netherlands), frozen in liquid nitrogen precooled isopentane and stored at −80 °C until immunohistochemical analysis. Healthy control skin samples ($n = 9$) were obtained from plastic surgery procedures or from routine circumcisions. Patients enrolled in this study did not receive treatment for their disease at the time of collection. All patients and healthy individuals provided informed consent and the procedures were carried out in accordance with the guidelines of the ethical committee of the Montpellier University Hospitals. Sections of cutaneous biopsies (6 μm) were obtained, using a cryomicrotome (Leica, Wetzlar, Germany), fixed with 3.7% formaldehyde/PBS for 10 minutes and incubated with 3% H_2O_2 in PBS for 10 minutes followed by blocking with PBS containing 0.1% gelatin, for 2 hours in a humidified chamber. Then sections were incubated overnight at 4 °C with the anti-CEACAM1 mAb 19.6 and the anti-CD3 mAb T3-4b5 (Dako SA, Trappes, France). Slides were processed for immunoperoxidase staining using the Dako ChemMate detection kit (Dako). Negative controls were carried out by omission of the primary antibody. Sections were then examined with a TE300 microscope equipped with a DMX1200 digital camera (Nikon, Tokyo, Japan). For the staining of monolayered NHEK, the cells were first cultured on coverslips and then processed as above. No permabilization step was applied in this protocol.

Western blotting analysis

NHEK were lyzed using Triton X-100 lysis buffer for 1 hour on ice. For western blotting analysis, protein extracts were boiled in SDS buffer, before their loading onto a 10% nonreducing SDS-PAGE gel. After electrotransfer to PVDF membranes (Millipore, Bedford, MA)

and blocking in saline buffer, containing 0.1% Tween-20 and 5% nonfat milk for 1 hour, blots were incubated overnight with the 19.6 or an anti-GAPDH mAb (Santa Cruz Biotechnology, Santa Cruz, CA). The presence of proteins was revealed by incubation with peroxidase-conjugated goat-anti-mouse IgG (ImmunoResearch Laboratories, West Grove, PA) and detection using the ECL advance western blotting detection kit (GE Healthcare, UK).

Measurement of production of cytokines and soluble CEACAM1

The presence of IFN-γ in culture supernatants was determined by cytokine-specific ELISA, as described (Pène *et al.*, 2006). Soluble CEACAM1 measurements were carried out according to a similar procedure, using the anti-CEACAM1 mAb 19.6 as the capture antibody ($2\,\mu g\,ml^{-1}$) and a biotinylated goat anti-recombinant CEACAM1 Ab (R&D Systems; BAF2244) as the detection antibody ($0.1\,\mu g\,ml^{-1}$). Streptavidin-AP (BD Pharmingen, Le-Pont-de-Claix, France; dilution 1/10,000) and the substrate 4-nitro-phenyl phosphate (Sigma-Aldrich) was used as the read-out. Recombinant CEACAM1 (R&D Systems) was used to establish a standard curve. The sensitivity of the assay was $300\,pg\,ml^{-1}$.

Analysis of neutrophil apoptosis

One million freshly isolated peripheral blood neutrophils were added to confluent layers of either NHEK, precultured in the absence or presence of $10\,\mu g\,ml^{-1}$ IFN-γ, to CEACAM1-L- or CEACAM1-S-expressing CHO cells or to nontransfected CHO cells, respectively, and the cells were cultured for various periods of time at 37 °C in a final volume of 1 ml. Alternatively, neutrophils were stimulated with the 19.6 mAb ($1\,\mu g\,ml^{-1}$), fMLP (10^{-8} M; Sigma-Aldrich) or GM-CSF ($200\,ng\,ml^{-1}$; R&D systems). Neutrophil apoptosis was measured by a procedure based on double-staining with FITC-Annexin-V (early apoptosis) and PI (late apoptosis), thus identifying early apoptotic cells as Annexin-V^+, PI^-, late apoptotic cells as Annexin-V^+, PI^+, and viable cells as Annexin-V^-, PI^- using the Bender Medsystems apoptosis kit (Tebu, Le Perray-en-Yvelines, France) on a FACSCalibur flow cytometer equipped with Cellquest software (BD Biosciences).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

Part of this study was supported by the Inserm Avenir Program, the Cancéropôle Grand-Ouest and by MedBioMed (Montpellier, France). M.R. was supported by MedBioMed and by the Region Languedoc-Roussillon, France.

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